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Original article

Innovative Strategy for Hydrocarbons Bioremediation Through Natural Enhancements

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Abstract
There are significant environmental issues that arise due to the existence of petroleum hydrocarbons, due to their persistence and toxicity. There is therefore a need for the development of sustainable techniques for bioremediation. This study focused on assessing the effects of biochar extracts, bile salts, and both treatments together in improving bacterial growth and hydrocarbon degradation processes. A total of five species of bacteria (Acinetobacter calcoaceticus, Rhodococcus ruber, Bacillus subtilis, Pseudomonas aeruginosa, and Mycobacterium gilvum) were cultivated in culture media that had diesel and motor oils at two different contamination levels (1:1 and 3:3 mg/mL). The bacterial growth process was monitored for 35 days, whereas hydrocarbon degradation was analyzed by the use of gas chromatography. It was found that bacteria grew in proportion to the increase in biochar extract and bile salt concentration, and that the Acinetobacter calcoaceticus showed the highest growth and efficiency in biodegradation. According to gas chromatography analysis, a significant decrease was found in both low and high-molecular-weight hydrocarbon fractions. It was found that the combination of biochar extract and bile salts produced higher efficiency in hydrocarbon degradation compared to any of the amendments used alone. This shows that there is a synergistic effect between the two amendments, which increases the bioavailability of the hydrocarbons.
Keywords. Biochar Extract, Bile Salts, Bioremediation, Hydrocarbon-Degrading Bacteria.

Introduction

Hydrocarbon pollution of the environment can be regarded as one of the most serious environmental issues due to the wide usage of hydrocarbons in various fields of activity, for various purposes. Contamination of the environment with petroleum products like diesel fuel, engine oil, and other derivatives of oil occurs due to leakage and spills in the process of storage, industry, and waste disposal. Due to their complex structure and hydrophobicity, these contaminants are persistent in soil and water environments. Once they get to the environment, they influence the fertility of the soil and soil microorganisms, restrict the growth of plants, contaminate water sources, and pose significant risks for the environment and humans due to their toxicity, mutagenicity, and carcinogenicity. This is why there is an urgent need for the development of relevant methods that will help deal with these environmental problems [1].

From among a broad range of remediation methods, bioremediation might be regarded as one of the most environmentally friendly ways of treating hydrocarbon-contaminated soil. In contrast to physical and chemical methods, which are usually costly and might result in additional pollution of the environment, bioremediation involves metabolic processes of microorganisms aimed at the conversion of petroleum hydrocarbons into harmless compounds, including carbon dioxide, water, and biomass. Decomposition of hydrocarbons by such microorganisms as Acinetobacter calcoaceticus, Rhodococcus ruber, Bacillus subtilis, Pseudomonas aeruginosa, and Mycobacterium gilvum, for instance, has proved to be highly efficient owing to the capacity of bacteria to decompose different kinds of hydrocarbons. Nevertheless, even if these microorganisms are highly efficient in the decomposition of hydrocarbons, some external factors influence the work of microorganisms [2].

In recent times, various approaches have been suggested to solve such issues, one of which is to utilize natural ingredients to promote the growth of microorganisms. This became possible due to the variety of qualities that biosurfactants and biochar have, and which will help to achieve efficient bioremediation without damaging the environment. Bile salts are biosurfactants that reduce the surface tension of water and increase the solubility of hydrophobic hydrocarbons, providing bacteria with the ability to access those compounds. Biochar is an organic substance characterized by high levels of carbon, which make it porous; besides, biochar improves the soil's capacity to retain nutrients and change its physical qualities. Moreover, biochar can adsorb harmful compounds from the soil. Therefore, bile salts and biochar separately have the potential to increase bioremediation. However, there has not been much research done on the effects of the synergetic actions of both substances [3].

While several experiments have proven the effectiveness of using biosurfactants and biochar alone in enhancing hydrocarbon biodegradation, there exist several issues that need to be addressed. This is because some studies are concerned with the effectiveness of one strain of bacteria under laboratory conditions, but there is scant information on the behavior of hydrocarbon-degrading bacteria under natural enhancement methods. Also, while studying the application of natural enhancement materials in bioremediation, most researchers concentrate on evaluating the microbial growth or hydrocarbon degradation, but very few studies have been done on microbial growth behavior and TPHs reduction simultaneously using techniques

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like gas chromatography. In addition, not much emphasis has been placed on determining the optimal amounts of natural enhancement that should be used to enhance bacterial activity [4].

The knowledge gap that has emerged from the current research is a result of inadequate information on how the two natural enhancers work in combination to improve the growth of the hydrocarbon degraders. While the separate effects of biochar and biosurfactants have already been established through previous research studies, there is not enough information on whether the use of both enhancement methods will be able to influence different hydrocarbon degraders using different concentrations of hydrocarbons, such as motor oil and diesel. There is a lack of information on how the two enhancement methods can influence the effectiveness of hydrocarbon degradation depending on the types of hydrocarbons [5].

This research seeks to overcome these limitations by providing an alternative approach to bioremediation by employing environmentally friendly natural enhancement, which will be done using a combination of biochar and bile salt extracts. The novelty of this research is shown through different approaches. First, the current research takes into account the synergic effects of two different natural enhancers on hydrocarbon biodegradation rather than the individual effects of the same. Second, the current research involves the comparison of five known hydrocarbon-degrading bacteria. This way, information about the efficiency of each of the bacteria under similar conditions is obtained. Third, the growth of bacteria and their biodegradation efficiency are analyzed at the same time using gas chromatography for measuring TPH. Fourth, the current research deals with different enhancement concentrations and hydrocarbon ratios [6].

The findings of this research will certainly contribute to the further development of environmental biotechnology as they will serve as the scientific evidence of the effectiveness of employing natural biosurfactants and biochar as eco-friendly enhancers. From the practical point of view, this method can be employed in relation to efficient cleaning of the oil-polluted soil and water areas. As the method employs natural enhancing methods, the related initiatives can fit into the framework of the greening practices and creation of the circular bioeconomy based on natural resources rather than chemicals.

Therefore, in this regard, the primary aim of the research project being conducted would be to investigate the efficiency of natural enhancing techniques, such as biochar and bile salt extracts, for hydrocarbon bioremediation. To do so, it is essential to perform research on the impact of various concentrations of biochar, bile salt extracts, and their mixture to assess their potential to improve the growth of bacteria and degradation of petroleum hydrocarbons in the diesel motor oil mixture.

Hydrocarbon pollution involves the occurrence of pollutants in different forms of the environment owing to the presence of pollutants that are formed by crude petroleum products. Hydrocarbons are organic substance that contains carbon and hydrogen as their main components and makes up most of the components in crude oil, gasoline, diesel, oils, and other petroleum products. Owing to the expansion of industries, petroleum exploration, transportation, mining activities, and manufacturing, there has been an increase in the number of cases of hydrocarbon pollution. There are several sources of hydrocarbon pollution, which include oil spills, leakage of underground storage tanks, wastewater, offshore drilling operations, and petroleum wastes [7].

Hydrocarbons in the environment will be present for quite some time due to the chemical stability of hydrocarbons and the problem that is associated with the degradation of hydrocarbons through the biodegradation process. Contaminants of the hydrocarbon type usually include aliphatic hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes. Among the four types of hydrocarbons, the Polycyclic Aromatic Hydrocarbons (PAHs) are more important and popular because of the properties of toxicity, persistence, mutagenicity, and carcinogenicity that are associated with them. The hydrophobic nature of the hydrocarbon molecules in petroleum leads to poor solubility of hydrocarbons in water [8].

Bioremediation of Petroleum Hydrocarbons

Bioremediation is another way of biological purification that entails the employment of living biological organisms, like bacteria, that break down the pollutants in the environment to environmentally friendly substances through biological processes. The hydrocarbon-oxidizing bacteria use the petroleum hydrocarbons as a carbon source and energy source; they then break down the complex hydrocarbons to simple products such as carbon dioxide, water, and bacterial cell masses. In comparison with other approaches employed in the clean-up of environmental pollutants, like digging of the soil, burning of the contaminated regions, and chemical oxidation, bioremediation is advantageous because of its eco-friendly nature and economic feasibility. The mechanism of biodegradation of the petroleum hydrocarbons requires different biochemical pathways facilitated by the presence of the enzymes known as oxygenases [9].

This type of organism is equipped with many types of metabolism, allowing degradation of alkanes, cycloalkanes, aromatic hydrocarbons, and polycyclic aromatic hydrocarbons. The bioremediation process requires many environmental variables such as temperature, pH, moisture, oxygen, nutrition, and the nature of contaminants. However, the primary limitation of bioremediation of petroleum contamination is that hydrophobic hydrocarbons are difficult to degrade as they stick to the soil. In natural attenuation, the native population of microorganisms is used, while in bioaugmentation, microbes that specialize in hydrocarbon decomposition are introduced to the contaminated area [10].

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Hydrocarbon-Degrading Bacteria

Hydrocarbon degraders are a type of microbe whose primary metabolic function entails the degradation of hydrocarbons found in petroleum through different metabolic pathways that depend on enzymes. It is not surprising for hydrocarbon-degrading bacteria to be naturally found in soils, sediments, freshwater, marine water, and petroleum deposits where there is hydrocarbon contamination. Metabolic abilities enable them to degrade many kinds of hydrocarbons, including short-chain alkanes, long-chain alkanes, monomers, and polycyclic aromatic hydrocarbons (PAHs). Degradation begins with the secretion of extracellular enzymes, such as monoxygenases and dioxygenases, which oxidize hydrocarbon compounds to intermediates. Intermediates are further metabolized by catabolic pathways [11].

There is variation in the degradation capabilities of each bacterial strain depending on the nature of the hydrocarbon and the prevailing conditions. For instance, Acinetobacter calcoaceticus is a species that utilizes aliphatic hydrocarbons and produces biosurfactants, allowing hydrocarbon bioavailability. The ability of Rhodococcus ruber to break down complex hydrocarbons is due to its hydrophobic cell membrane and numerous catabolic enzymes. Bacillus subtilis produces the biosurfactant surfactin that makes it possible to emulsify hydrocarbons using microorganisms. Pseudomonas aeruginosa has become extensively studied because it produces biosurfactant rhamnolipids capable of metabolizing hydrocarbons extracted from petroleum. At last, Mycobacterium gilvum proves very effective in breaking down high molecular weight hydrocarbons and aromatic hydrocarbons resistant to breakdown [12].

Biochar

Biochar can be described as an inactive material that is highly concentrated with carbon and which is produced by means of the pyrolysis of organic matter with minimal oxygen content. There are various sources for biochar production, such as agricultural waste, forestry waste, animal waste, and organic sources. Properties like high porosity, large specific surface area, and abundant functional groups make biochar extensively investigated and applied to the areas of environmental remediation, carbon capture, and soil amendment. Recently, biochar has been discovered to enhance microbial bioremediation of oil-contaminated soils [13].

The studies conducted have indicated that biochar improves the efficiency of bioremediation of hydrocarbons as a result of its unique physicochemical properties. Firstly, the porosity of biochar promotes the formation of biofilms by the bacteria capable of breaking down hydrocarbons. Secondly, the application of biochar leads to improved soil aeration, water retention, cation exchange, and nutrient availability, thus providing an optimum environment for microbiological processes. Besides, biochar acts as a sorbent to remove any toxic compounds and intermediates that could inhibit bacterial activity. In the case of contaminated soils with hydrocarbons, biochar indirectly helps in improving the process of biodegradation since it stimulates microbial growth and enzyme production; however, it does not degrade hydrocarbons itself [14].

Bile Salts

The bile salt is a mixture that exists naturally and is synthesized from cholesterol in the liver, and is very important for fat digestion in the body. The molecules of bile salt have both hydrophobic and hydrophilic regions, and hence, bile salt functions as a biosurfactant and lowers the surface tension of oil-water emulsions. The physical property of bile salts makes them commonly used in environmental biotechnology, mainly as surfactants in bioremediation of hydrophobic compounds like petrochemicals [15].

One of the principal problems encountered during the petroleum bioremediation process is related to the low solubility of hydrocarbons in water and their bioavailability. Hydrophobic contaminants tend to aggregate or attach themselves to soil components, making it difficult for the microorganism to come into contact with the contaminant. This is where the function of bile salt is important due to its capability to decrease the interfacial tension between hydrocarbons and water and enhance their bioavailability. Unlike other synthetic surfactants, bile salts offer many environmental advantages. They have a high level of biodegradability, in addition to having a relatively low toxicity and persistence in the environment [16].

Methodology

The methodology used in this study was experimental laboratory research that assessed the efficiency of using natural enhancing agents in the bioremediation of petroleum hydrocarbons. The experiment method was chosen since this method allows the independent variable to be manipulated while its effects on hydrocarbon degradation are assessed in laboratory conditions. The experiment was done using a Completely Randomized Design (CRD), where there were four treatment groups, including a control group with no enhancement agents, a biochar extract treatment, a bile salt extract treatment, and a treatment that used both biochar extract and bile salt extract. There were five different hydrocarbon degrading bacteria species included in each treatment, which were Acinetobacter calcoaceticus, Rhodococcus ruber, Bacillus subtilis, Pseudomonas aeruginosa, and Mycobacterium gilvum. Each treatment was done in triplicate to guarantee the reliability and reproducibility of the experiment. The efficiency of the treatment was measured by measuring bacterial growth during the incubation process and the reduction of Total Petroleum Hydrocarbon (TPH) by Gas Chromatography with Flame Ionization Detector (GC-FID).

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The laboratory experiments were conducted in the Environmental Microbiology Lab, where bacterial cultivation, inoculum preparations, and biodegradation experiments were done under controlled environmental conditions. The analysis of degradation of petroleum hydrocarbons was done in the Analytical Chemistry Lab through Gas Chromatography-Flame Ionization Detection (GC-FID). All the laboratory practices were in line with the institutional guidelines on biosafety and laboratory safety for accurate analysis.

The materials included hydrocarbon-degrading bacteria cultures, petroleum hydrocarbons, natural enhancers, microbiological media, reagents, and laboratory instruments. Five bacterial species with the proven ability to degrade hydrocarbons were chosen as the biological agents, such as Acinetobacter calcoaceticus, Rhodococcus ruber, Bacillus subtilis, Pseudomonas aeruginosa, and Mycobacterium gilvum. The bacterial cultures were purchased from recognized microorganism culture collections and cultured on Nutrient Agar before the experiment. Contamination by petroleum was performed through the use of commercial diesel fuel and used motor oil, which are commonly found as hydrocarbon contaminants in the polluted environment. Biochar extract was the first natural enhancer, which was made by the pyrolysis of agricultural biomass in an oxygen-free environment. For the second enrichment medium, bile salt extract was prepared using analytical-grade bile salts in distilled water. Mineral Salt Medium (MSM) was used as the base medium since it contains inorganic nutrients while forcing the bacteria to metabolize hydrocarbons as the only source of carbon. Other materials used include Nutrient Broth, Nutrient Agar, distilled water, n-hexane for the extraction of hydrocarbons, and analytical-grade chemicals for microbiological and chromatographic analysis.

All experiments were done in triplicate, and the data generated were presented as mean values along with the corresponding standard deviation. At first, descriptive statistical analysis was carried out to give an overview of the growth of bacteria and hydrocarbon degradation under various conditions. Inferential statistical analysis was further carried out using SPSS version 29.0.

Result and Discussion

This chapter discusses the results of the experimental study conducted to investigate the effectiveness of natural enhancing materials in the bioremediation process of petroleum hydrocarbons using selected bacteria that can degrade hydrocarbons. In the experiment, the impacts of varying concentrations of bile salt extracts and biochar extracts were studied on the growth of five types of bacteria, which include Acinetobacter calcoaceticus, Rhodococcus ruber, Bacillus subtilis, Pseudomonas aeruginosa, and Mycobacterium gilvum. Growth of the bacteria was measured for 35 days under the influence of two mixtures of hydrocarbons, namely diesel and motor oils. The results will be shown to indicate the changes in growth rates due to the application of different enhancement techniques.

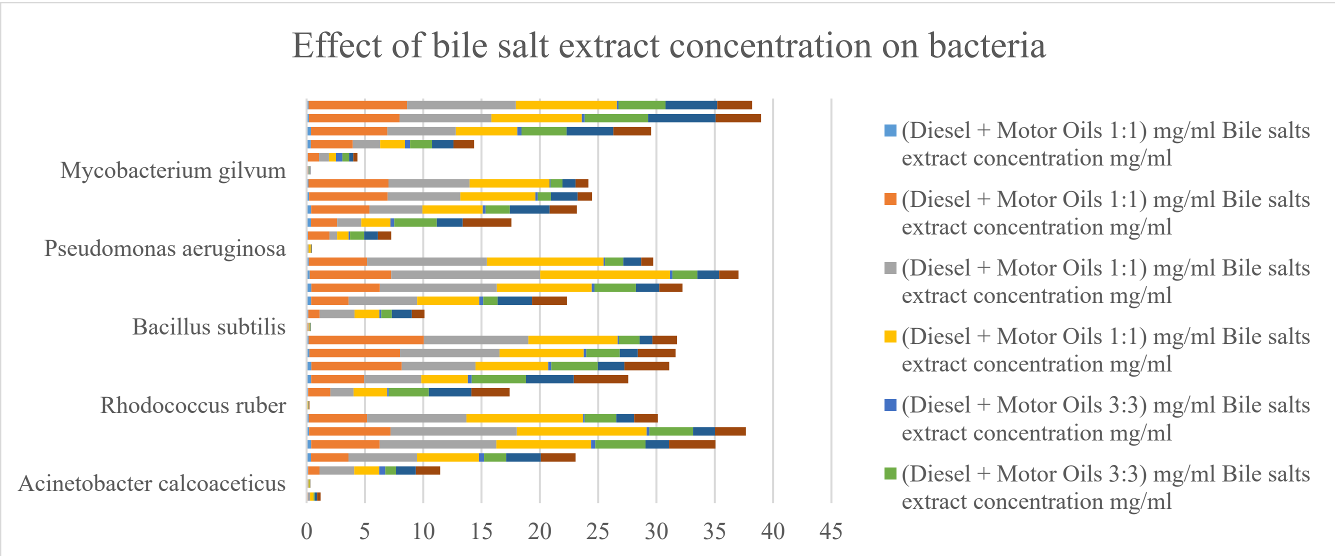

Figure 1. Results Of The Effect Of Bile Salt Extract Concentration On Bacteria Growth

Figure 1 shows the bacterial growth patterns in response to various amounts of bile salt extracts for two kinds of hydrocarbons, which are the mixtures of diesel and motor oil with ratios 1:1 and 3:3. In general, there is an increase in bacterial growth over time for all bacterial strains due to adaptation to contaminated medium with petroleum. Bacterial growth in the presence of bile salt extract was higher than that of the control, thus showing an increase in the bioavailability of hydrocarbons.

At a ratio of diesel and motor oil 1:1, the maximum bacterial growth occurred at bile salts at 0.2 and 0.3 mg/mL. The bacteria that showed maximum growth were Bacillus subtilis, which had a value of 12.769 on Day 28 at the level of 0.2 mg/mL. Likewise, Acinetobacter calcoaceticus showed maximum growth with a

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value of 11.130 on Day 28 at 0.3 mg/mL, while Mycobacterium gilvum showed maximum growth with a value of 9.344 on Day 35 at 0.2 mg/mL. Furthermore, there was maximum growth improvement in Rhodococcus ruber and Pseudomonas aeruginosa compared to the control, although this improvement was different depending on the level of bile salt and incubation period. Bacterial growth in the control was minimal throughout the experiment.
The growth rate of bacteria under the more concentrated mixture of hydrocarbons (diesel and motor oil 3:3) was lower than the growth rate in the case of a 1:1 mixture, implying that higher hydrocarbon concentration put more stress on the bacteria's metabolism. However, even in such conditions, bile salt addition still improved the growth rate of bacteria compared to the control condition. As for the tested bacteria, Acinetobacter calcoaceticus and Mycobacterium gilvum appeared to be more adapted to the higher hydrocarbon concentration, while the growth of Pseudomonas aeruginosa was lower in later incubation periods.

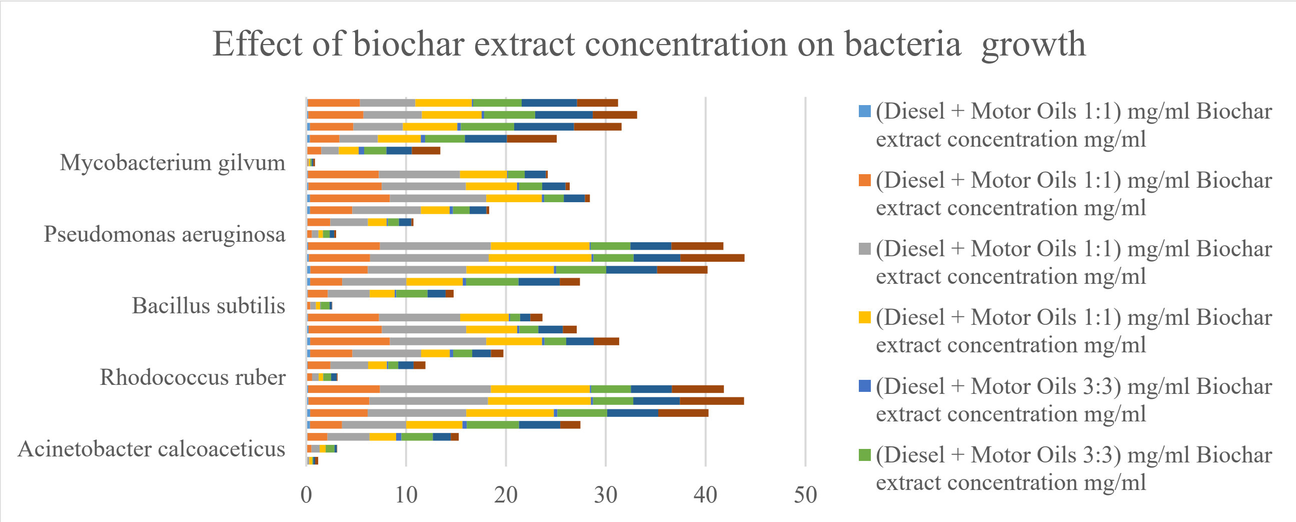

Figure 2. Results Of The Effect Of Biochar Extract Concentration On Bacteria Growth

Bacterial growth after biochar extract treatment at different concentrations in the two hydrocarbon compositions is shown in Figure 2. Bacterial growth steadily increased with time for most bacterial species just like the effect of the bile salt treatments. This clearly shows that biochar created a conducive environment for bacteria to colonize and metabolize the hydrocarbons. The biochar treatments resulted in greater bacterial growth than in the absence of biochar.
In the case of the mixture of diesel and motor oil in the ratio of 1:1, maximum bacterial growth was seen at the concentration of biochar of 0.2 mg/mL. The maximum optical density of Acinetobacter calcoaceticus was recorded as 11.876 on Day 28, whereas Bacillus subtilis also showed a maximum optical density of 11.875 in the same manner. Rhodococcus ruber and Pseudomonas aeruginosa also reacted positively with biochar and gave their maximum optical densities as 9.652 and 9.655, respectively, on Day 21 in the 0.2 mg/mL concentration of biochar. On the other hand, despite low growth in comparison with the other bacteria, the growth of Mycobacterium gilvum also increased with an increase in the concentration of biochar and gave its maximum optical density of 5.989 on Day 28.
The bacterial growth in the case of the diesel and motor oil mixture of 3:3 was still less than the bacterial growth recorded in the case of the 1:1 ratio because of the higher petroleum content that acts as an inhibitor. Nevertheless, biochar application still enhanced bacterial growth when compared to the control. Out of the five bacterial strains studied, Mycobacterium gilvum showed high adaptation to the high hydrocarbon content, reaching almost 6.0 OD value. The bacteria Acinetobacter calcoaceticus and Bacillus subtilis showed relatively high growth, while Rhodococcus ruber and Pseudomonas aeruginosa were slightly better off. It can be concluded that the biochar extract positively affects the growth of hydrocarbon-utilizing bacteria through creating suitable habitats, retaining nutrients, and providing favorable environmental conditions for them. In addition, it should be noted that the higher effectiveness at the intermediate concentration means that further increase of biochar amount will not necessarily improve bacterial growth proportionally.

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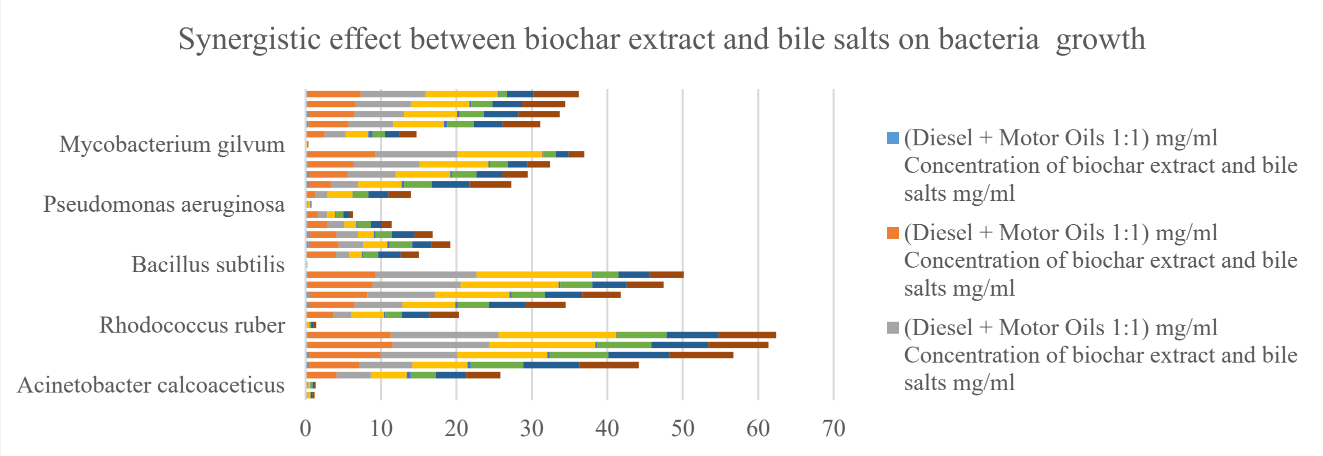

Figure 3. Results Of The Synergistic Effect Between Biochar Extract And Bile Salts On Bacteria Growth

Figure 3 shows the synergic effect between biochar extract and bile salts on the growth of five hydrocarbon degraders in diesel-motor oil mixes at 1:1 and 3:3 ratios for 35 days. Generally, growth rates increased as the concentration of the two additives increased, suggesting that biochar extract and bile salts had synergic effects in promoting the growth of microbes. Positive impacts started to be shown after the first week of the incubation period. This could mean that the microbes needed some time to adapt to use hydrocarbons as energy sources. Of the five microbes, Acinetobacter calcoaceticus showed the highest growth rate in the 1:1 hydrocarbon ratio, reaching 15.653 at 0.3 mg/mL for 35 days.
Similar results were recorded for Rhodococcus ruber, where the increase in growth continued to increase until the end of the experiment, when the final value was 15.298. Pseudomonas aeruginosa and Mycobacterium gilvum also showed growth that is dependent on the concentration, but the maximum biomass was lower than that of Acinetobacter calcoaceticus and Rhodococcus ruber. On the other hand, Bacillus subtilis showed another growth pattern where there was an increase in the growth for the first few days and then a decrease after day 21.
Even though bacteria grew less under the more concentrated hydrocarbon mixture (3:3) than under the 1:1 hydrocarbon mixture, their growth was still improved in all cases where biochar and bile salts were added to the cultures. This shows that these amendments helped the bacteria to adapt better to the conditions and grow effectively even in such concentrations of hydrocarbons. It is possible that these amendments facilitated hydrocarbon utilization due to the surfactant properties of the biochar extract and created a favorable microenvironment for bacterial growth.

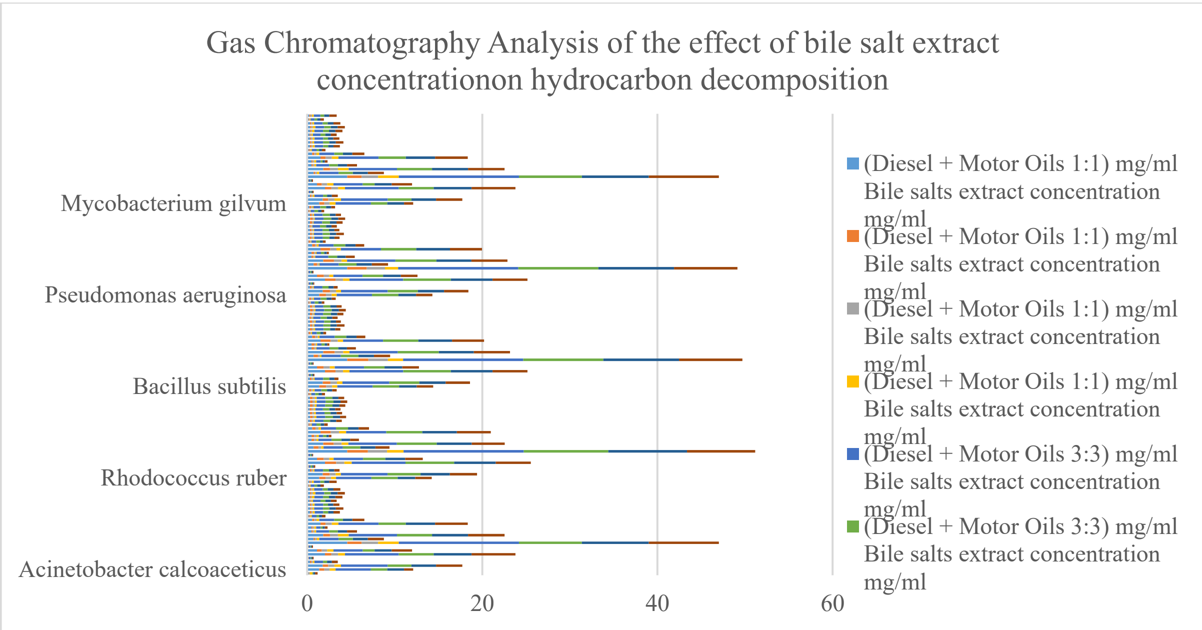

Figure 4. Result Gas Chromatography Analysis Of The Effect Of Bile Salt Extract Concentration On Hydrocarbon Decomposition

Figure 4 shows the GC analysis of the degradation of hydrocarbons treated with different amounts of bile salts in diesel and motor oils mixture (1:1 and 3:3). Generally, the residual levels of both low-molecular

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weight (C7–C12) and high-molecular weight (C16–C32) hydrocarbons were decreased in proportion to the increase in the amount of bile salts; therefore, it can be said that the use of bile salts increased the rate of degradation of hydrocarbons by microorganisms. More significant decreases in residual hydrocarbons occurred in the 1:1 mixture than in the 3:3 mixture of hydrocarbons, meaning that lower amounts of contaminants helped microorganisms to degrade them. Among all tested bacterial species, Pseudomonas aeruginosa and Rhodococcus ruber showed the best results. These microorganisms formed the lowest level of residual hydrocarbons at 0.3 mg/mL bile salts, especially in C16–C21 hydrocarbons, which are hard to degrade by microorganisms because of higher molecular weight and water insolubility.
Acinetobacter calcoaceticus and Mycobacterium gilvum also displayed high degradation rates; however, the remaining hydrocarbon quantities were higher than those found for Pseudomonas aeruginosa. On the other hand, Bacillus subtilis displayed lower degradation rates when compared to other bacteria during the process in both hydrocarbon mixtures, showing that it is less tolerant or capable of degrading hydrocarbons under the same conditions. Higher hydrocarbon degradation rates due to increased bile salt concentration can be explained by the surfactant action of bile salts, which make hydrocarbons more soluble and emulsifiable. As a result, substrate availability for bacteria becomes higher, and bacteria become more efficient in their work.

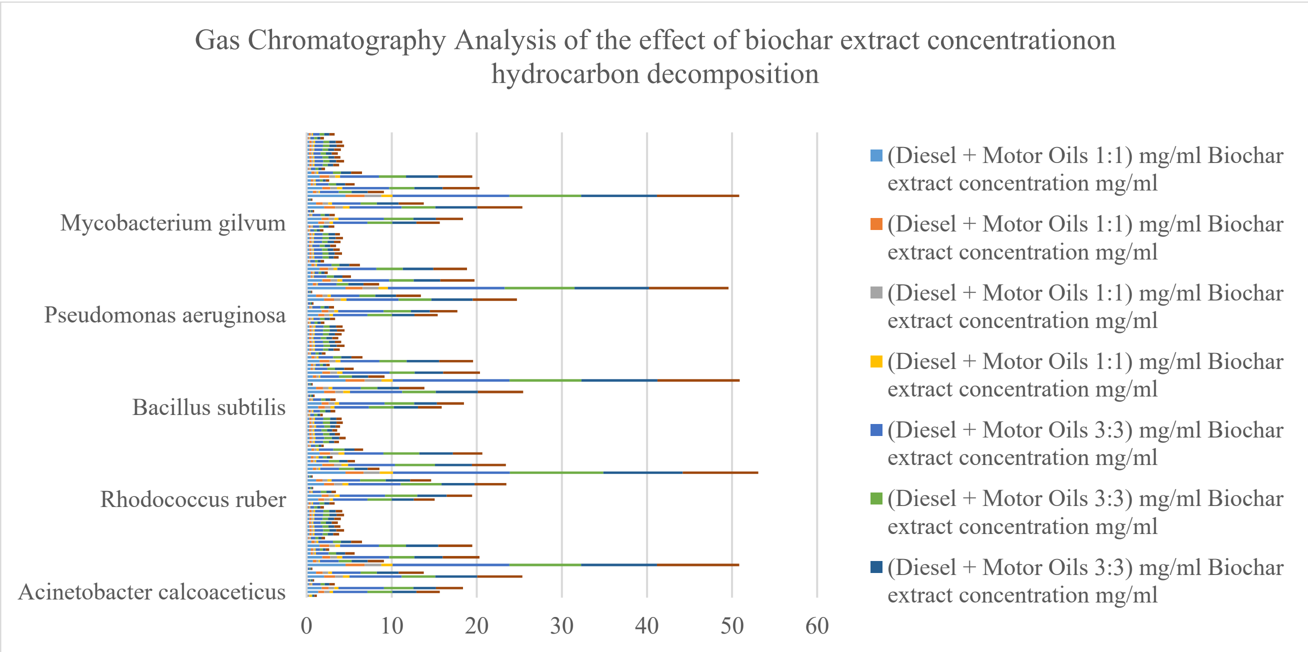

Figure 5. Result Gas Chromatography Analysis Of The Effect Of Biochar Extract Concentrationon Hydrocarbon Decomposition

The figure below describes the results of gas chromatography on hydrocarbon degradation after exposure to varying concentrations of biochar extract. It can be seen that there is an increase in hydrocarbon degradation efficiency with the increase in biochar extract concentration, but this varies depending on the type of bacteria and hydrocarbon mixture used. The concentration of all hydrocarbons was gradually decreasing from the control up to 0.3 mg/ml of biochar extract concentration, showing that there is an increase in biodegradation efficiency based on concentration. This was especially observed in diesel and motor oil (1:1) hydrocarbon mixture because of the ability of Pseudomonas aeruginosa and Acinetobacter calcoaceticus to demonstrate low residual concentration of medium and long chain hydrocarbons (C12–C21).
Furthermore, Rhodococcus ruber proved to be an efficient degrader, especially in regard to high molecular weight hydrocarbons, while Bacillus subtilis was found to possess medium efficiency. With regard to the more concentrated mixture of diesel and motor oil (3:3), the rate of degradation was lower compared to the 1:1 treatment due to the presence of a larger amount of hydrocarbons, though there were also reductions in hydrocarbons with an increase in biochar concentration in comparison with the control sample. It is reasonable to suppose that the effect of improved degradation in the presence of biochar can be explained by its porous nature, which gives appropriate conditions for the bacteria to attach while also absorbing the toxic substances capable of inhibiting bacterial activity.

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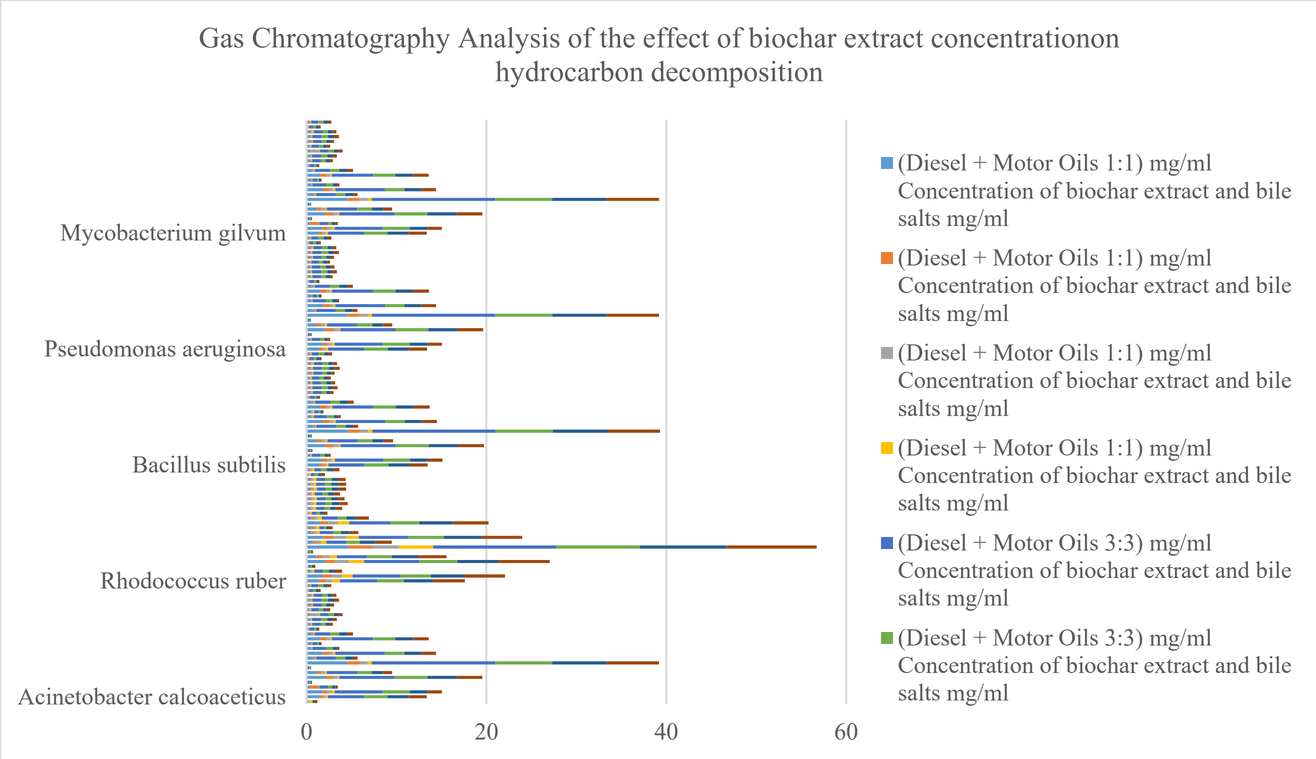

Figure 6. Result Gas Chromatography Analysis Of The Effect Of Biochar Extract Concentrationon Hydrocarbon Decomposition

The results of the gas chromatography analysis for hydrocarbon degradation after the combined use of biochar extract and bile salts are depicted in Figure 6. In comparison to the separate use of each amendment analyzed in Tables 4 and 5, the synergic use resulted in the highest percentage decrease in residual hydrocarbons for almost all carbon fractions, indicating the presence of a strong synergy between the two additives. For both hydrocarbon mixtures, there was a tendency towards progressive reduction of residual concentrations with the increase in the concentration of the combined mixture from the control level up to 0.3 mg/mL, proving that the simultaneous action of biochar extract and bile salts was very effective for the biodegradation of petroleum hydrocarbons. The largest amount of degradation was demonstrated by Pseudomonas aeruginosa and Acinetobacter calcoaceticus, which had the lowest levels of residual hydrocarbons in both short-chain (C7-C12) and long-chain (C16-C32) hydrocarbon mixtures at a concentration of 0.3 mg/mL.

Similarly, the Bacillus subtilis strain performed better in degradation than either biochar alone or bile salts alone, but still had inferior effectiveness in comparison to the Pseudomonas aeruginosa strain. On the contrary, the Rhodococcus ruber strain showed a lesser reaction to the combined treatment, meaning that there might be differences in how biochar and bile salts interact based on the bacteria's physiology and metabolic reactions. The increased efficiency of the combined treatment can be explained by the interaction of both materials used as amendments. Bile salts provide emulsification of the hydrocarbons and their availability, while biochar increases the surface for attachment of bacteria and decreases the toxicity of hydrocarbons, in addition to stabilization of the microenvironment. All these factors contribute to better efficiency of hydrocarbon degradation, thus making the combined use of biochar extract and bile salts the best option for petroleum hydrocarbon biodegradation among all other treatments.

Discussion

Synergistic Effect of Biochar Extract and Bile Salts on Bacterial Growth

The current experiment sought to determine whether the use of natural additives, such as biochar extract and bile salts, can stimulate the growth of bacteria responsible for the degradation of hydrocarbons in the process of biodegradation of crude oil. When biochar extract and bile salts were used together, a substantial increase in bacterial growth was registered within the 35-day incubation period, as compared with the control group. It should be noted that the growth was directly related to the concentration level, and the highest bacteria population was observed in the case of 0.3 mg/mL [17].

Of all the isolates investigated in this research, Acinetobacter calcoaceticus and Rhodococcus ruber were identified to have the most significant increase in growth, especially when the combination of diesel and motor oil (1:1) was used. These bacteria are known hydrocarbon degraders since they can feed on aromatic and aliphatic hydrocarbons to obtain carbon and energy. The improved growth observed in this case indicates that the combination of biochar extract and bile salt made hydrocarbons more bioavailable and

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reduced the environmental stress caused by the toxicity of the hydrocarbons. Bile salts act as natural surfactants that break down hydrophobic hydrocarbons, thereby increasing their dispersion. Biochar, on the other hand, is characterized by its porous nature and large surface area that facilitates microbial attachment, moisture retention, and absorption of toxic materials [18].
All bacterial strains grew positively in response to the combination treatment; however, variations in growth indicated different metabolic abilities inherent to each particular strain. For instance, Pseudomonas aeruginosa and Mycobacterium gilvum demonstrated increasing growth at different stages of incubation, which proved their ability to survive under hydrocarbon conditions. In turn, Bacillus subtilis had rather small growth in the later stages of incubation, indicating poor adaptation to hydrocarbons or inability to utilize them due to less complex metabolic pathways. These results are quite natural since hydrocarbon decomposition is possible only with catabolic enzymes [19].

Effect of Bile Salts on Hydrocarbon Degradation
The gas chromatographic analysis indicated that bile salts greatly improved petroleum hydrocarbon degradation in all bacterial strains tested, thus confirming the validity of the initial research hypothesis. The increase in bile salt concentration from the control to 0.3 mg/mL was characterized by decreasing concentrations of residual hydrocarbons in both short and long chain fractions. These results imply that the availability of hydrophobic hydrocarbons in microbial metabolism became higher under the influence of bile salts. Since petroleum hydrocarbons have very poor water solubility, the lack of bioavailability of these compounds becomes a limiting factor for their biodegradation. The emulsification property of bile salts eliminates this factor [20].
Pseudomonas aeruginosa and Rhodococcus ruber showed the highest degradation efficiency and consistently had the lowest amounts of hydrocarbon residue at the highest level of bile salt content. Both of them have a wide range of oxygenase enzymes, capable of initiating oxidation reactions in a variety of petroleum hydrocarbons. They can utilize emulsified substrates effectively. The high degradation ability was also shown by Acinetobacter calcoaceticus and Mycobacterium gilvum; however, their residues were slightly larger compared to those from Pseudomonas aeruginosa. On the contrary, the degradation ability of Bacillus subtilis was comparatively low [21].
The greater residue hydrocarbon concentrations found in the diesel and motor oil (3:3) combination reveal that increased contaminant concentrations have decreased the rate of hydrocarbon degradation even with the presence of bile salts. Increased petroleum concentration might increase toxicity, decrease the availability of oxygen, and impair enzyme activity in microorganisms, leading to reduced rates of hydrocarbon degradation. However, bile salts were still able to enhance the rate of hydrocarbon degradation when compared to the control group without any treatments. The observed reduction of both high and low-molecular-weight hydrocarbons implies that bile salts helped degrade a broad range of hydrocarbons instead of only degradable ones [22].

Effect of Biochar Extract and the Combined Application of Biochar Extract and Bile Salts on Hydrocarbon Degradation
The results from Tables 5 and 6 confirm that the use of biochar extract alone contributed to petroleum hydrocarbon degradation, whereas their combined effect with bile salts was the best degradation efficiency compared to all other treatments used. An increase in the biochar amount led to a continuous decrease in hydrocarbon concentration, hence showing that biochar facilitated the growth of microbes and hydrocarbon metabolism. Nevertheless, the use of biochar extract alongside bile salts increased the efficiency of hydrocarbon degradation, thus confirming that both amendments work in synergy. This is in line with the objectives of this research, which focused on the evaluation of natural methods of enhancing bioremediation of petroleum products [23].
Biochar is involved in the degradation of hydrocarbons through various complementary actions. Firstly, biochar offers huge surfaces for microbial adhesion, thus allowing biofilm formation and making bacteria stable during long incubations. Additionally, biochar absorbs toxic components of petroleum products, preventing their inhibitory effect on bacteria, while providing better soil aeration and water content. As a result, bacteria remain active for a prolonged period of time and are able to use hydrocarbons effectively. Such factors provide the explanation of the progressive decrease in the amount of hydrocarbons depending on the increase of biochar content in mixtures of hydrocarbons [24].
The combination therapy resulted in further improvement in degradation since the bile salts improved the emulsification and solubility of hydrocarbons, whereas the biochar offered a stable site for bacterial colonization. The complementary action between the two agents made it easier for the bacteria to utilize the substrate and kept them active throughout the degradation process. Pseudomonas aeruginosa and Acinetobacter calcoaceticus responded best to the combination therapy, having recorded the least amount of hydrocarbons in nearly all the carbon fractions. It can be attributed to the fact that they are known to have enzyme systems capable of degrading hydrocarbons and good adaptability to polluted environments. Despite the positive impact of the combination therapy on Bacillus subtilis, its degradation capacity was relatively poor [25].

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Conclusion

The current study has shown that the natural enhancing methods involving the use of extracts from biochar, bile salts, and their mixture had great effects on the bacterial growth and biodegradation of hydrocarbons in diesel and motor oil contaminated samples. The bacterial isolates performed well in terms of growth when they were mixed with an adequate amount of biochar and bile salts, though the extent of this effect was dependent on the specific microorganisms. Acinetobacter calcoaceticus grew and degraded efficiently as compared to Pseudomonas aeruginosa and Mycobacterium gilvum, whereas Bacillus subtilis had poor biodegrading ability. The gas chromatography tests revealed considerable TPH fraction reductions.
Out of all the treatments applied, it was noted that the use of biochar and a combination of bile salts resulted in the best results with regard to the removal of hydrocarbons, which shows that there is a synergy between them that improves microbial activity and increases the availability of hydrocarbons. In fact, this combination has effectively decreased both the short-chain and long-chain hydrocarbons in a mixture of diesel and motor oils, proving that this treatment is more effective compared to the use of only biochar or bile salts. Based on this experiment, it can be seen that the hypothesis that the use of natural additives can increase microbial degradation of petroleum hydrocarbons is correct.

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